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A simple and cost-effective method to prepare template DNAs for cell-free protein synthesis

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Abstract−The scale-up of cell-free protein synthesis reactions involves the preparation of large amounts of template DNA. While ion-exchange column chromatography methods have commonly been used to obtain purified plasmid DNA for cell-free protein synthesis reactions, these methods are costly and difficult to expand to a large scale. In this work, we report that the routine isopropyl alcohol (IPA) precipitation method can be used to prepare cell-free-expressible DNA when the co-precipitated proteins are removed. Compared to column-purification procedures, the IPA-precipitation offers obvious advantages with respect to the cost and scaling-up of template preparation, and we believe that our finding will contribute to making cell-free protein synthesis system more practical for the rapid production of preparative amounts of recombinant proteins.

Key words: Plasmid, Cell-free Protein Synthesis, Scale-up, Extraction

INTRODUCTION

As the demand for high-throughput protein expression increases due to recent progress in many genome sequencing projects, cell-free protein synthesis has received considerable attention as an attractive alternative to conventional *in vivo* gene expression technology [1]. Since cell-free protein synthesis techniques do not require the cultivation of living cells, target proteins can be obtained with minimal processing time, labor and laboratory set-up. Moreover, recent advances in the understanding of the cell-free metabolism of key substrates have enabled the development of more efficient and economical methodologies for cell-free protein synthesis [2-6]. For example, Calhoun and Swartz demonstrated that expensive secondary energy sources (phosphoenol pyruvate, creatine phosphate or acetyl phosphate) could be replaced with glucose [7], which provides for the regeneration of ATP by glycolytic enzymes present in the cell-free extract. Since the cost of glucose is almost negligible compared to the previously used energy sources, this finding greatly improved the economical feasibility of cell-free protein synthesis. In parallel, the procedures for the preparation of translational machinery have also been greatly simplified and improved. In particular, Kim et al. recently reported a simplified method for the preparation of the cell-extract, which could be completed within 2 hours involving neither high-speed centrifugation nor dialysis steps [8]. Combined together, these will remarkably improve the feasibility of cell-free synthesis technology as an alternative for the rapid production of recombinant proteins on a preparative scale.

On the other hand, not much attention has been paid to the preparation of template DNA for cell-free protein synthesis. Although ion-exchange column purification methods are widely used to prepare the plasmid templates, the column-based plasmid purification steps are costly and laborious.

Alcohol-mediated precipitation is a simple and inexpensive technique for the crude preparation of plasmid DNA [9], and it would substantially improve the feasibility of large-scale cell-free protein synthesis reactions if alcohol-precipitated plasmids could be used directly as templates. In this study, we demonstrate that isopropyl alcohol (IPA)-precipitated plasmids can direct cell-free protein synthesis as efficiently as column-purified plasmids after an appropriate work-up procedure.

Direct incubation of the plasmid prepared by simple IPA-precipitation failed to produce detectable amounts of proteins in our cell-free synthesis system. We discovered that this was due to RNase(s) co-precipitated with plasmid, and inactivation of the contaminating RNase(s) by Proteinase K enabled the IPA-precipitated plasmid to direct protein synthesis. As a more economical method to eliminate the RNase activity, phenol extraction of the IPA-precipitated plasmid was as effective as Proteinase K treatment. Quantitative analysis showed that the yield of protein synthesis from the crude plasmid treated in these ways was at least equal to that from column-purified plasmids. Our finding will substantially contribute to the construction of cell-free protein synthesis systems for commercial purposes.

MATERIALS AND METHODS

1. Materials

ATP, GTP, UTP, CTP, creatine phosphate (CP), and *E. coli* total tRNA mixture were purchased from Roche Applied Science (Indianapolis, IN). L-[U-^{14}C]leucine (11.9 GBq/mmol) was obtained from Amersham Biosciences (Uppsala, Sweden). All other reagents were purchased from Sigma and used without purification. T7 RNA polymerase and S30 extract were prepared according to previously reported protocols [10].

2. Plasmid Preparation

All of the plasmids examined in this study were amplified in the *E.*

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coli strain JM109. Transformed cells were cultivated overnight in 200 ml of LB medium, harvested, and lysed in 5 ml of SDS/NaOH solution [14]. After being neutralized with 5 ml of potassium acetate (3 M, pH 5.5), the lysate was centrifuged and the supernatant was recovered.

For alcohol-assisted precipitation of DNA, the supernatant was mixed with an equal volume of isopropyl alcohol (IPA). After 10 min at room temperature, the precipitated DNA was recovered by centrifugation (15,000 RCF, 30 min). The plasmid pellet was then washed with cold ethanol (80%, v/v), dried under vacuum and re-dissolved in TE buffer.

To remove the protein species contaminating the IPA-precipitated plasmid by Proteinase K, the re-dissolved plasmid solution was incubated with 20 μg/ml of Proteinase K (Invitrogen, Carlsbad, CA) at 65 °C for 10 min and subsequently the enzyme was inactivated at 90 °C for 10 min.

Otherwise, cleared cell lysate was extracted with phenol prior to the IPA-precipitation step. 500 μl of lysate was mixed with an equal volume of phenol (equilibrated with Tris-HCl, pH 8.0) and mixed vigorously in a micro-centrifuge tube. After centrifugation (14,000 RCF for 5 min), the aqueous phase was withdrawn and mixed with two volumes of pure ethanol to precipitate the plasmid. The precipitate was recovered by centrifugation, rinsed with 70% ethanol, dried under vacuum, and re-dissolved in 100 μl of deionized water. Highly purified plasmid for the control reaction was prepared by using a column purification kit according to the manufacturer's instructions (Maxiprep, Qiagen, Valencia, CA).

3. Cell-free Protein Synthesis Reaction

The standard reaction mixture for the coupled transcription/translation consisted of the following components in a total volume of 15 μl: 57 mM Hepes/KOH, pH 8.2, 1.2 mM ATP, 0.85 mM each of GTP, UTP, and CTP, 1.7 mM dithiothreitol, 80 mM ammonium acetate, 33 μg/ml T7 RNA polymerase, 0.17 mg/ml total tRNA mixture (from strain MRE 600), 34 μg/ml l-5-formyl-5,6,7,8-tetrahydrofolic acid (folinic acid), 1.5 mM each of the unlabeled amino acids (5.6 μg/ml), 0.3 U/ml creatine kinase, 67 mM creatine phosphate, and 4 μl S30 extract. Depending on the experiment, 0.1 μg plasmid was used as the template to direct the protein synthesis. The synthesis reactions were conducted in a water bath at 37 °C for 2 h. The synthesized protein was quantified by measuring the TCA-precipitable radioactivity as described previously [10-13]. The molecular weight of the expressed protein was confirmed with a 13% Tricine-SDS-polyacrylamide gel [15] and Western blot analysis.

4. *In Vitro* Transcription and Analysis of mRNA

In vitro transcription reactions were conducted by incubating the prepared plasmids in a reaction mixture identical to the one for cell-free protein synthesis, except that the S30 extract and exogenous tRNA mixture were not added. Reaction samples were withdrawn during the incubation of the reaction mixture and immediately mixed with equal volume of RNA Protect Bacteria™ reagent (Qiagen, Valencia, CA). The mRNA in each sample was then extracted and purified using an RNeasy Mini kit (Qiagen, Valencia, CA). The size of the isolated mRNA was analyzed on a 2% formaldehyde agarose gel as described earlier [16].

RESULTS AND DISCUSSION

While ion-exchange columns are commonly used to isolate and purify plasmids, the column-based purification procedures are expensive and cannot be readily expanded to large-scale preparation. In addition, although the purity of DNA often critically affects the performance of many molecular biological applications, considering that the current cell-free synthesis systems employ crude cell extract as the source of translational machinery, it does not seem reasonable to add highly purified plasmids to cell-free protein synthesis reactions that already contain a crude mixture of cellular proteins.

Alcohol-mediated precipitation is one of the simplest methods for the isolation of DNAs, and has long been used to prepare crude plasmid DNA prior to further purification steps. In order to develop a cost-effective and scalable method to prepare the template for cell-free protein synthesis, we examined whether plasmids that had been prepared by IPA-precipitation could be used directly for cell-free protein synthesis. After alkaline lysis of the overnight-grown cells that was followed by the neutralization step, the plasmid pIVEX2.3-EGFP encoding the enhanced green fluorescent protein (EGFP) was isolated by adding an equal volume of IPA to the cleared lysate. The prepared plasmid was analyzed on 1.5% agarose gel, and was added to the reaction mixture for cell-free protein synthesis. After 2 hours of incubation at 37 °C, the expression of EGFP in the reaction mixture was analyzed by Western blot as described in Materials and Methods. As shown in Fig. 1, direct use of the IPA-precipitated plasmid did not generate protein, while the control reaction using column-purified plasmid successfully produced the target protein.

In addition, when the IPA-precipitated plasmid was incubated in an *in vitro* transcription reaction (Fig. 2(a)), no transcripts were de-

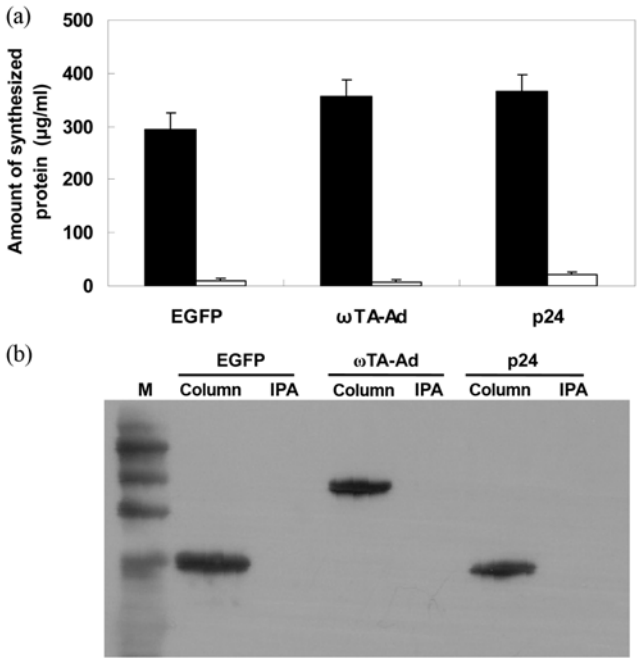

Fig. 1. Effect on the efficiency of protein synthesis by different methods of plasmid preparation. Cell-free protein synthesis reactions were carried out using column-purified (filled bars) or IPA-precipitated (open bars) plasmids as the reaction templates. Amounts of the expressed protein were estimated by measuring the TCA-insoluble radioactivities after 2 h of incubation (panel (a)). Samples were also analyzed by Western blot (panel (b)).

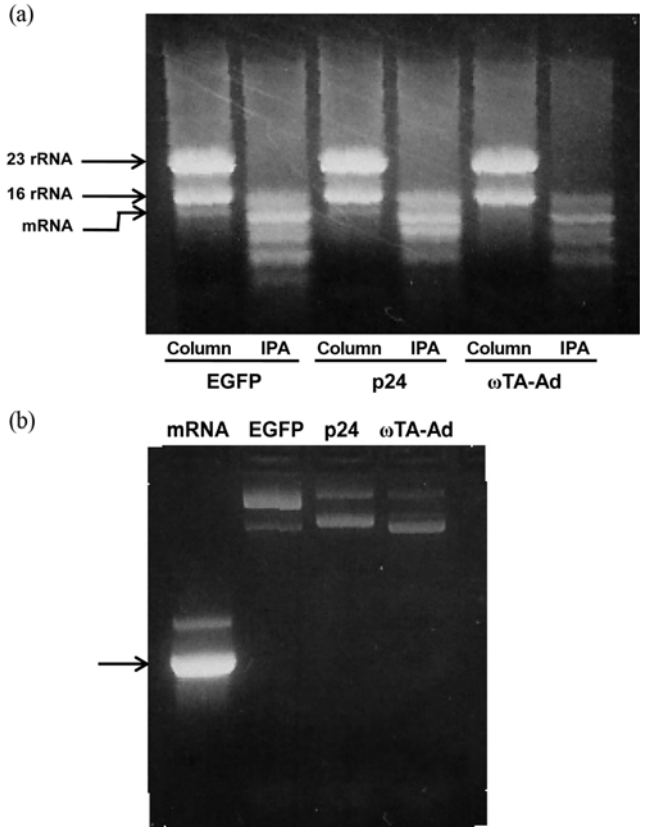

Fig. 2. Degradation of mRNA in the presence of IPA-precipitates. (a) IPA-precipitated or column purified plasmids (pIVEX2.3-EGFP, pIVEX2.3-ωTA-Ad and pIVEX2.3-p24) were incubated in a reaction mixture for cell-free protein synthesis. 5 μl of reaction samples was withdrawn after 40 min and residual RNAs were analyzed on a 2% formaldehyde agarose gel. (b) mRNA of EGFP was prepared by *in vitro* transcription of the plasmid pIVEX2.3-EGFP as described in Materials and Methods. Purified mRNA (5 μg/ml) was then mixed with water (lane C) or IPA-precipitated plasmids (lane 2, pIVEX2.3-EGFP; lane 3, pIVEX2.3-ωTA-Ad; lane 4, pIVEX2.3-p24), and incubated 10 min at 37 °C. 5 ml of the incubated samples were analyzed on a 2% formaldehyde agarose gel.

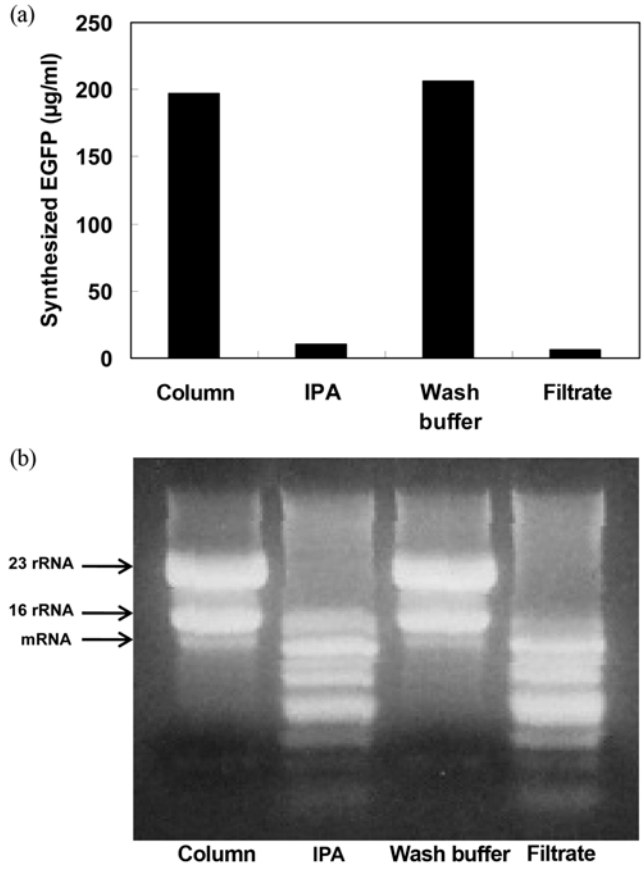

Fig. 3. The filtrate from the washing step inhibited protein synthesis. (a) pIVEX2.3-EGFP was incubated in a reaction mixture containing a wash buffer (marked as "wash buffer") or the filtrate from washing step (marked as "filtrate"). After 2 h of incubation, 2 μl of the reaction mixture was isolated and analyzed by Western blot analysis. (b) After 40 min incubation, 5 μl of the reaction mixture was isolated and analyzed by denaturing electrophoresis on a 2% formaldehyde agarose gel.

tected. Furthermore, co-incubation of the IPA-precipitates with purified mRNA caused rapid degradation of the mRNA (Fig. 2(b)). Taking these results together, we concluded that the IPA-precipitated plasmid was contaminated with a substantial amount of RNase activity, which repressed cell-free protein synthesis through degradation of the transcripts.

In contrast, when the IPA-precipitated plasmid was loaded onto an ion exchange column and subsequently washed with the provided buffers, the eluted plasmid was able to direct cell-free protein synthesis. Therefore, it appeared that the column purification steps effectively removed the RNase activity from the IPA-precipitated plasmid. Typical procedures for ion-exchange column purification of plasmids consist of three steps: binding, washing and elution. We collected the filtered buffer solutions at each step and added them back to a complete reaction mixture for cell-free protein synthesis in order to determine which fraction exhibited RNase activity. As a result, while the filtrate from the binding step did not affect protein synthesis (data not shown), the filtrate from the washing step (marked as "filtrate") substantially inhibited protein synthesis (Fig. 3(a)). Similar to the results in Fig. 2, the inhibition of protein synthesis stemmed from degradation of the mRNA (Fig. 3(b)). Therefore, we assumed that the endogenous RNases bound to the ion-exchange resin and were effectively separated from the plasmid during the wash steps. SDS-PAGE analysis of the filtrates showed that numerous protein species were eluted in the wash step (data not shown), and we are currently working to identify the protein that is responsible for deteriorating the efficiency of cell-free protein synthesis.

Based on the assumption that contaminating RNases are responsible for the incompetence of the IPA-precipitated plasmids with cell-free protein synthesis, we sought other methods to remove or to inactivate RNase(s) than the column-purification protocols. We thus examined if the IPA-precipitated plasmid could direct protein synthesis when the contaminating proteins were removed. Indeed, when the IPA-precipitated plasmid was treated with Proteinase K as described in Materials and Methods, successful expression of the

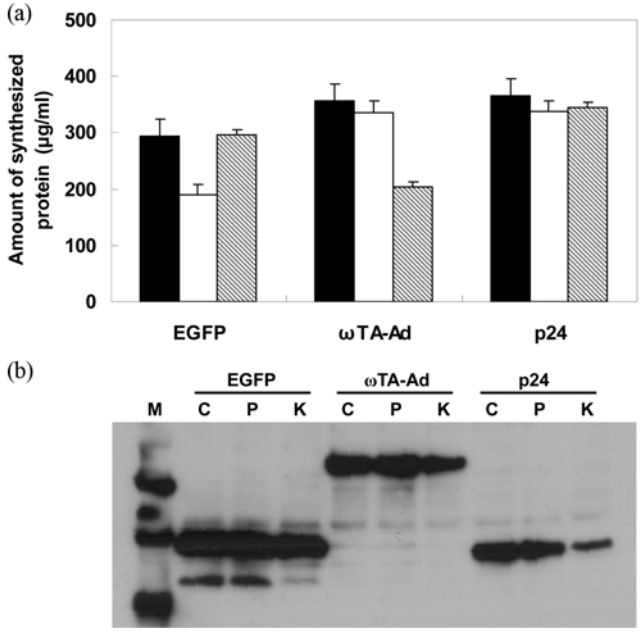

Fig. 4. Cell-free expression of IPA-precipitated plasmids after phenol extraction or proteinase K treatment. Plasmids prepared by IPA-precipitation procedures were incubated in a reaction mixture for cell-free protein synthesis after being treated with ion-exchange column (filled bars, C), phenol (open bars, P) or proteinase K (slashed bars, K). After being incubated for 2 h at 37 °C, 15 μl samples were withdrawn and TCA-insoluble radioactivity was measured as described in Materials and Methods. 2 μl samples were analyzed by Western blot.

target proteins was observed, indicating that removal of the contaminating RNase activity is critical for the IPA-precipitated plasmid to direct protein synthesis (Fig. 4). Subsequently, as a more economical method to remove the contaminating proteins, the aqueous solution of crude IPA-precipitate was treated with phenol as described in Materials and Methods, and the recovered DNA was incubated in the reaction mixture for cell-free protein synthesis. Again, the use of phenol-extracted plasmid led to successful generation of the encoded protein (Fig. 4). Quantitative analysis of the expressed protein (by measuring the TCA-insoluble radioactivity as described in Materials and Methods) indicated that the amount of protein expressed from the phenol-extracted plasmid was comparable to that from the column-purified DNA. The results of the Western blot analysis of the cell-free synthesized proteins also suggest that the IPA-precipitated plasmids were of comparable 'quality' to the column purified plasmids when the contaminating RNase activity was removed as described. It should be noted that the cell-free protein synthesis system employs crude cell extract (S30 extract) as the source of translation machinery. Therefore, the RNase activity that is responsible for the degradation of template mRNA should be present in the reaction mixture for cell-free protein synthesis, even prior to the addition of the IPA-precipitated plasmid. It appears that the inhibition of protein synthesis by the RNase activity becomes apparent due to its concentration during the IPA-precipitation procedures. Eventually, specific identification of the responsible RNase and its removal at the genetic level would be the most desirable for plasmid preparation in the construction of a large-scale cell-free protein synthesis system.

CONCLUSIONS

Availability of a simple and cost-effective method for the preparation of DNA templates becomes of great importance when a scaling-up of cell-free protein synthesis is considered. We demonstrated that plasmids prepared by routine IPA-precipitation method were able to direct efficient cell-free protein synthesis when the contaminating RNase activity was removed by phenol extraction or proteinase K treatment. While the process time and cost for plasmid preparation can be substantially reduced by this method, compared with the conventional column purification procedures, the efficiency of protein synthesis from the resulting plasmids was not compromised. We believe that the presented results will contribute to making the technology of cell-free protein synthesis more economical and scalable.

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